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Research paper

Infectious titres of human papillomaviruses (HPVs) in patient lesions, methodological considerations in evaluating HPV infectivity and implications for the efficacy of high-level disinfectants

Michelle A. Ozbun[a,b,c,*], Virginie Bondu[a], Nicole A. Patterson[a], Rosa T. Sterk[a],
Alan G. Waxman[b], Erica C. Bennett[d], Rohini McKee[e], Ankur Sharma[f], Jeremy Yarwood[f],
Marc Rogers[f], Gary Eichenbaum[g]

[a] Department of Molecular Genetics & Microbiology, The University of New Mexico School of Medicine, Albuquerque, NM 87131, United States
[b] Department of Obstetrics & Gynecology, The University of New Mexico School of Medicine, Albuquerque, NM 87131, United States
[c] The University of New Mexico Comprehensive Cancer Center, Albuquerque, NM 87131, United States
[d] Department of Surgery, Division of Otolaryngology-Head and Neck Surgery, The University of New Mexico School of Medicine, Albuquerque, NM 87131, United States
[e] Department of Surgery, Division of General Surgery, The University of New Mexico School of Medicine, Albuquerque, NM 87131, United States
[f] Advanced Sterilization Products, Inc., 33 Technology Drive, Irvine, CA 92618, United States
[g] Johnson & Johnson, Office of the Chief Medical Officer, 410 George Street, New Brunswick, NJ 08901, United States

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ABSTRACT

Background: Recent publications from a single research group have suggested that aldehyde-based high-level disinfectants (HLDs), such as *ortho*-phthalaldehyde (OPA), are not effective at inactivating HPVs and that therefore, patients may be at risk of HPV infection from medical devices. These results could have significant public health consequences and therefore necessitated evaluation of their reproducibility and clinical relevance.

Methods: We developed methods and used standardised controls to: (1) quantify the infectious levels of clinically-sourced HPVs from patient lesions and compare them to laboratory-derived HPVs, (2) evaluate experimental factors that should be controlled to ensure consistent and reproducible infectivity measurements of different HPV genotypes, and (3) determine the efficacy of select HLDs.

Findings: A novel focus forming unit (FFU) infectivity assay demonstrated that exfoliates from patient anogenital lesions and respiratory papillomas yielded infectious HPV burdens up to 2.7×10^3 FFU; therefore, using 2.2×10^2 to 1.0×10^4 FFU of laboratory-derived HPVs in disinfection assays provides a relevant range for clinical exposures. RNase and neutralising antibody sensitivities were used to ensure valid infectivity measures of tissue-derived and recombinant HPV preparations. HPV infectivity was demonstrated over a dynamic range of $4-5 \log_{10}$; and disinfection with OPA and hypochlorite was achieved over 3 to $>4 \log_{10}$ with multiple genotypes of tissue-derived and recombinant HPV isolates.

Interpretation: This work, along with a companion publication from an independent lab in this issue, address a major public health question by showing that HPVs are susceptible to HLDs.

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1. Introduction

Human papillomaviruses (HPVs) are highly transmissible nonenveloped viruses that cause significant health and economic burden worldwide [1]. HPV infections are spread by close contact and cause epithelial warts and papillomas with some infections progressing to malignancies [2]. HPV DNA and/or RNA can be detected on medical devices [3–5], equipment, and surfaces in clinical settings [6], and is commonly described as "viral load". However, the relationship between viral nucleic acid load and HPV infectious potential has not been previously evaluated.

Questions about the effectiveness of high-level disinfectants such as *ortho*-phthalaldehyde (OPA) against laboratory-produced HPVs

have arisen following publications from a single research group [7–9]. The prospect that certain commonly-used disinfection approaches may not be effective at inactivating HPV infectivity contradicts the accepted hierarchy of microbial resistance to biocides [10] and merits thorough examination. To investigate the relationship between HPV DNA load in lesions and infectious potential and to comprehensively assess the efficacy of different disinfectants against HPV, infection assays were carefully controlled and novel methods were developed. To our knowledge, these approaches facilitated, for the first time, evidence for the levels of infectious HPV in clinical lesions to which medical instruments might be exposed. We quantified and compared infectious levels of clinically-sourced HPVs from patient lesions to laboratory-derived HPV levels used in *in vitro* assays, and evaluated the factors that must be controlled to ensure consistency and reproducibility for *in vitro* HPV infectivity assessments. We then utilised these well-controlled methods to determine the efficacy of disinfectants such as OPA and hypochlorite in inactivating multiple HPV genotypes isolated by common experimental approaches.

2. Methods

2.1. Ethics

The collection of clinical material used in this study complied with the Helsinki Declaration of 1975, as revised in 1983 and described previously and was approved by the University of New Mexico Health Sciences Center's Human Research Protections Office (UNM HRPO #17-202 IRB Approved Protocol).

2.2. Papilloma sampling and HPV typing

Between August 2017 and April 2018, subjects with papillomas consistent with HPV infection were approached to participate in the study. After receiving written, informed consent, physicians rubbed the lower half of a small (1 × 4-cm), sterile piece of emery paper (600A-grit Wetordry Tri-M-ite; 3M) 5 times across the surface of the papilloma, similar to a study wherein HPV DNA prevalence was tested [11]. Exfoliates were eluted with sterile PBS. Briefly, each emery paper swab was held at the upper half with sterile tweezers, with the lower half in a 1.7 mL microcentrifuge tube. PBS (250 μl) was used to rinse the exfoliate from the lower portion of the paper, rinsing 2−3 times into the microcentrifuge tube. The rinse was repeated with a second 250-μl aliquot of PBS for a total recovery volume of 0.5 ml. The PBS clinical virion stocks were stored at 4 °C. HPV DNA typing was performed according to manufacturer's instructions using the SEEGENE HPV28 assay (Anyplex II HPV28 detection kit, Seegene). This multiplexed genotyping system is semiquantitative and facilitates simultaneous detection and identification of 19 high-risk HPVs (16, 18, 26, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 66, 68, 69, 73, 82) and 9 low-risk HPVs (6,11, 40, 42, 43, 44, 54, 61, 70). For infectivity comparison to laboratory-derived HPV stocks, clinical samples positive for HPV11, HPV16 and/or HPV31 were subject to quantification of viral genome equivalents (VGE) by quantitative PCR (qPCR).

2.3. Viral genome equivalent (VGE) quantification of virus stocks

All HPV virion preparations were quantified for encapsidated viral genomes using TaqMan qPCR specific to the HPV long control region (LCR), a noncoding segment of the viral genome, as previously described [12,13]. HPV stocks were diluted (e.g., 1:50 and 1:500) in carrier DNA (sheared salmon sperm DNA [Invitrogen] or human placental DNA [Millipore Sigma]) at a carrier concentration of 50 ng/ul. qPCR reactions were performed using 5 μL of the 1:50 and 1:500 virus stocks using primers targeted to the HPV LCR (Bio-Rad SsoFast EvaGreen Supermix) (see Supplemental Table 2). For each assay a calibration curve spanning 10^8-10^0 genome copies was run in triplicate and was generated by serial dilutions of cloned HPV genomes. Data were collected on a Bio-Rad CFX96 and analysed using Bio-Rad CFX Manager (version1.6.541.1068). Only experiments with calibration

curve R^2 values of ≥ 0.995 and amplification efficiencies between 90 and 100% were included.

2.4. Cell lines

The cell lines W12 (RRID:CVCL_T290) and CIN-612 were established from human cervical intraepithelial neoplasia grade I (CIN1) biopsies [14,15]. The clonal CIN-612 9E cell line (RRID:CVCL_ER27; a gift of L. Laimins, Northwestern Univ.) maintains episomal HPV31 (subtype 31b) genomes at $\approx 50-100$ copies per cell [16]. W12 clone 20863 (W12-E) cells (gifted by P. Lambert, Univ. of Wisconsin-Madison and M. Stanley, Univ. of Cambridge) harbour episomal HPV16 genomes at ≈ 1000 copes per cell [17]. CIN-612 9E and W12-E cells were grown in monolayer culture in the presence of mitomycin C-treated J2 3T3 mouse fibroblast feeder cells (RRID:CVCL_W667) with E medium containing 5% fetal bovine serum (FBS) and 100 U/ml Nystatin (Sigma-Aldrich) as previously described [12]. HEK-293T cells (RRID:CVCL_0063) obtained from C. Buck (U.S. National Cancer Institute) were maintained in DMEM (Gibco Cat. # 11965-084). The HaCaT cell line (RRID:CVCL_0038), a gift of N. Fuesnig (DKFZ), are a spontaneously-immortalized, HPV-negative epithelial line derived from normal adult skin [18]. HaCaT cells were maintained in DMEM/F12-Ham's Nutrient Mixture containing 4X amino acids, 2 mM L-glutamine, 100 U/ml penicillin, and 1 ug/ml streptomycin (each from Sigma) and supplemented with 10% FBS (Atlanta Biologicals). Cell lines were authenticated by short tandem repeat analysis (IDEXX) and used within 15 passages of verification; mycoplasma screening was performed once per month.

2.5. Human papillomavirus production and isolation

Infectious HPV virion stocks were obtained from three laboratory model systems, including virion biosynthesis in mouse xenograft tissues and organotypic (raft) epithelial tissue cultures, and the recombinant assembly of quasivirions (QV). HPV11 virions from infected human keratinocyte xenografts grown in athymic mice as previously described [19] were a gift of Neil Christensen (Penn State College of Medicine). In short, the xenograft-derived HPV11 stock was obtained as a supernatant following mincing and disruption of infected tissue by Virtis homogenization in PBS at 4 °C at 25,000 rpm. for 30 min. Supernatants and pellets were separated by sedimentation at 11,000 x g. HPV16 and HPV31 virions were acquired from organotypic (raft) epithelial cultures [12]. HPV16 and HPV31 virions were acquired from organotypic (raft) epithelial cultures grown from W12-E and CIN-612 9E cells, respectively, as previously described [12,20]. For the raft-derived HPV31 stock described here, 80 raft tissues were cultivated to obtain the virus titres reported. Two methods were used to isolate virus particles from the raft tissues. First, crude virus preparations (CVPs) were obtained as described by Conway et al., wherein 5-10 raft tissues were homogenized in sodium phosphate solution (1M NaCl/0.05 M Na-Phosphate buffer, pH 8.0) using a Dounce apparatus and large cellular debris were pelleted [21]. The CVP supernatant was collected and stored at −20 °C for short-term storage. Second, a more refined raft virus preparation was obtained as previously detailed [12,22]. Briefly, raft tissues were homogenized using a BeadBeater™ tissue grinder. After a series of low- and high- speed centrifugation steps to remove cellular debris, DNA-containing virus particles were pelleted based on a sedimentation coefficient of 296S−300S for viral DNA-containing particles [23]. Recombinant "quasivirion" (QV) production was modified from that previously published [24]. Briefly, cloned viral genomes were released from plasmids by restriction endonuclease digestion. Intramolecular ligations were performed, and DNA was concentrated by isopropanol precipitation. HEK-293T cells were co-transfected with the re-circularized HPV genome, and a genotype-matched, codon optimized HPV L1- and L2- expressing plasmid (see Table 1). At 72 h post-

transfection, the cells were lysed and treated with 99% pure Benzonase (EMD Millipore) and Plasmid-Safe ATP Dependent DNase (Epicentre). Virions were incubated at 37 °C for 24 h to permit capsid maturation [25]. Cellular debris were pelleted, and the supernatants were subjected to a 1.25−1.4 g/mL CsCl step gradient and centrifugation at 20,000xg for 18 h. The opalescent viral band was extracted by side puncture, virions were washed 2−3X against Virus Stabilization Buffer (VSB) (25 mM HEPES pH 7.5, 500 mM NaCl, 1 mM MgCl$_2$) and concentrated using an Amicon Ultra-4 centrifugal filter 100 K (Millipore).

All virion stocks were quantified by qPCR based upon VGE content as noted above [12,13]. When virus stock yields allowed, SDS-PAGE and Coomassie Brilliant Blue staining were used to assess purity and presence of appropriately sized L1 (≈ 55 kDa) and L2 (≈ 72 kDa) capsid proteins (Suppl. Fig. 1). The virus stock concentrations of L1 and L2 proteins were determined by Chemidoc analytic software (Bio-Rad) and comparison to a BSA standard curve.

2.6. Infectivity assays

HaCaT cells were seeded at 1.5×10^5 cells per well into 12-well tissue culture plates and incubated overnight at 37 °C, 5% CO$_2$ prior to infections. The clinically sourced HPV11- and HPV16-positive virus isolates with the highest physical viral genome titres were freeze-thawed three times and sonicated prior to exposure to cells. HaCaT cells at $\approx 70\%$ confluency were exposed to virions in the absence or presence of genotype-specific neutralising antibodies [12] for up to 48 h at 37 °C with 5% CO$_2$. Cells were lysed in Trizol (Sigma) and RNA was extracted per the manufacturer's instructions. Reverse transcription (RT) of a constant amount of total RNA, typically 30 μg, with random hexamer primers (Invitrogen), dNTPs (Invitrogen), RNase inhibitor (Applied Biosystems) and M-MLV reverse transcriptase (Invitrogen) in 20-μl reactions was performed at 37 °C for 60 min. Quantitative polymerase chain reaction (qPCR) was performed using 10X PCR buffer II and AmpliTaq Gold (Applied Biosystems) in triplicate with 5.0 μl of cDNA using primers and probes in Suppl. Table 3; ß-actin was amplified as a reference control for RT efficiency as previously reported [26]. A calibration curve with copy number controls spanning 10^8-10^0 were prepared by serial 10-fold dilution from genotype specific, cloned HPV E1^E4 cDNA. Amplifications were in 96-well plate format on a on a Bio-Rad CFX96 and analysed using Bio-Rad CFX Manager (version1.6.541.1068). To be considered a valid qPCR reaction, the R^2 values were ≥ 0.995 and amplification efficiencies were 90−100% for each assay.

RNAscope in situ hybridization (RNA-ISH; Advanced Cell Diagnostics [ACD]) of viral E6/E7 mRNAs[27] was adapted as a focus forming assay to detect HPV infected cells. HaCaT cells were seeded into 8-well chamber slides at 5×10^4 cells per well and exposed to virus stocks. NIKS-SG3 cells persistently infected with HPV16 [28] were used as a positive control and were treated with 20 mg/ml RNase A to verify the specificity of E6/E7 mRNA detection by RNA-ISH. RNAscope was performed with HPV-HR7 E6/E7 probes (ACD Cat. #312351), specific for HPV types 16, 18, 31, 33, 35, 52 and 58, or HPV-LR10 E6/E7 probes (ACD Cat. #314551), recognizing HPV types 6,11, 40, 43, 44, 54, 69, 70, 71 and 74. Stained slides were subject to high-definition digital imaging using Aperio Image Scope microscopy (Leica). Cells were considered to be positive when there were ≥ 2 punctate signals indicative of HPV E6/E7 mRNA detection. Infrequently, a single punctate signal was observed in a cell, which was considered to be non-specific staining. Positive cells were counted by eye from digitized images.

2.7. Virus neutralization procedures

Genotype specific anti-HPV monoclonal antibodies [29], anti-HPV11 clone 11F.G1, anti-HPV16 clone H16.V5, anti-HPV31 clone

H31.A6, (a gift of N. Christensen, Penn State College of Medicine) were incubated with HPV virion stocks at 37 °C for 1 h prior to exposure to cells. Chemical disinfection was performed using a modified suspension test method[30] (Suppl. Fig. 2). Herein, one volume of virions (5 μL) was incubated with nine volumes of either 80% Cidex (final 0.44% OPA) or 10% bleach (final 0.825% hypochlorite [ClO-]) diluted in complete HaCaT medium for 12 min at RT. This 10^{-1} dilution of treated virus was combined with 450 μL of the appropriate neutralizer (1% or 7% v/v glycine for OPA; 2% v/v sodium thiosulphate [$Na_2S_2O_3$] for hypochlorite), yielding a 10^{-2} virus dilution. Untreated controls included virus incubated with 1X VSB; HaCaT medium alone was considered the neutralizer for the virus/buffer positive control. The virus/disinfectant was subject to four 10-fold serial dilutions (10^{-3}–10^{-6}) in the appropriate neutralizer and incubated 15 min, RT. HaCaT cells were exposed to the virus-antibody or virus-disinfectant solutions and allowed to incubate for up to 48 h at 37 °C with 5% CO_2. Cells were harvested for RNA; for experimental consistency, 3.0 μg of DNase I-treated RNA from each infection was subject to RT (total 20 μl reaction) and three 5.0-μl technical cDNA replicates were assayed for HPV E1^E4 mRNA levels by qPCR as described above. ß-actin cDNA was amplified as an internal RT control.

2.8. Statistical analyses

Data were analysed using GraphPad Prism 8 software, including simple linear regression and two-tailed Student's *t* test, which was used to compare differences between two groups. All data are presented as means ± standard deviation (SD). A *p* value of <0.05 was considered statistically significant and *p* values are shown in the figures.

2.9. Role of the funders

The study sponsors had no role in the collection, analysis, and interpretation of data or in the decision to submit the paper for publication. The U.S. NIH had no role in the study design or the writing of the report. ASP had a limited role in consulting on disinfection experimental design and in the writing the report.

3. Results

3.1. HPV genome loads in patient papillomas

Exfoliates from the surfaces of seven RRP and seven anogenital papillomas positive for HPV11, HPV16 and HPV31 were quantitatively assessed for encapsidated VGE (Fig. 1, Table 1). Total HPV11 recovered from the RRPs and one anal wart ranged from 3.3×10^4 to 1.4×10^7 VGE. Total HPV31 from the RRP samples ranged from 2.0 to 4.0×10^4 VGE. Samples from three HPV16-positive anal warts (AW) were assayed to have HPV16 DNA loads ranging from 2.5×10^5 to 3.9×10^8 VGE; one also had HPV31 at 3.3×10^5 VGE. Lacking definitive means to verify complete endonuclease digestion of free viral genomes, these VGE load measures may be over-estimates of that encapsidated within viral particles.

3.2. Impact of viral preparation on HPV infectivity across different virus types

In a prior report, vast differences in disinfection susceptibility were observed when comparing crude HPV16 preparations (CVP) derived from raft epithelial tissues with recombinant HPV16 QV refined by gradient centrifugation [7]. Yet, the differing virus isolation methods could introduce confounding variables, such as free viral nucleic acids, if not properly controlled. Predominantly, HPV virion isolation methods have utilised whole epithelial tissue homogenisation followed by a series of low and high-speed centrifugations to separate virions from unpackaged nucleic acids and cellular debris [22] (see Supplemental Methods). Previous disinfection studies employed a CVP of raft-derived HPV wherein homogenised tissues were simply clarified using low-speed centrifugation [21]. Therefore, we directly compared physical and infectivity characteristics amongst HPV virions obtained from the common laboratory models: xenograft tissues, organotypic (raft) epithelial tissues, and the recombinant QV assembly system. Salient characteristics of laboratory-derived virus stocks are summarised in Table 2 and are highlighted below.

Experiments were undertaken to verify *bona fide* infection from each laboratory-derived virus stock using the established criteria of time-dependent infection measures and neutralisation of infection
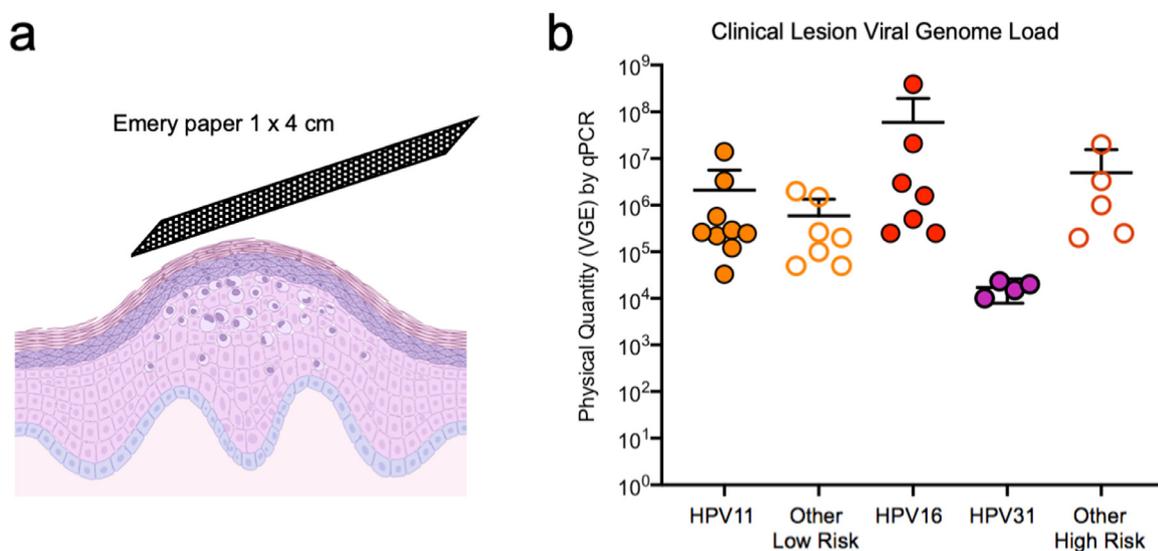
Fig. 1. Clinical lesion sampling and quantification of HPV genome load from samples. (a) Exfoliating cells and squames were collected from the surface of clinically-diagnosed HPV lesions using five passes of a sterile emery paper (see Supplemental Information). Image made in collaboration with BioRender. (b) DNA was isolated from exfoliated cells from atop clinically-diagnosed papillomas and subject to genotyping. Samples positive for HPV11, HPV16 and/or HPV31 were assessed for genotype specific viral DNA load in triplicate by qPCR. Also shown are the estimated sum levels of genome copies per sample for non-HPV11 low risk types (types 6, 43, 44, 54, 61, 70) or non-HPV16/31 high-risk types, (types 33, 35, 39, 53, 56, 59, 68). Bars represent the mean values of total VGE from each sample; mean values and 95% CI are shown. Details of cases are shown in Table 1.

Table 1
HPV genotypes, genome loads and titres from clinical lesions.

Sample[a]	HPV genotype(s) (semiquantitative DNA load)[b]	Total HPV genome load (VGE)[c]	Physical titre (VGE/mL)	Infectious by RT-qPCR	VGE per FFU[d]	Infectious units (FFU) per sample[e]
RRP 1	**LR: 11 (+++)**	HPV11−1.4×10^7	2.7×10^7	Yes	5.6×10^3	2.5×10^3
RRP 2	**LR: 11 (+++)**	HPV11−1.2×10^5	2.3×10^5	N.D.[f]	N.D.	N.D.
	HR: 31 (+)	HPV31−3.0×10^4	6.0×10^4	N.D.		N.D.
RRP 3	**LR: 11 (++)**	HPV11−2.2×10^5	4.5×10^5	N.D.	N.D.	N.D.
RRP 4	**LR: 11 (+++)**	HPV11−2.9×10^5	5.8×10^5	N.D.	N.D.	N.D.
RRP 5	**LR: 11 (++)**	HPV11−5.7×10^5	1.1×10^6	N.D.	7.6×10^3	7.5×10^1
	HR: 31 (+)	HPV31−2.0×10^4	4.0×10^4	N.D.	N.D.	N.D.
RRP 6	**LR: 11 (++)**	HPV11−2.6×10^5	5.3×10^5	N.D.	N.D.	N.D.
RRP 7	**LR: 11 (+++)**, 43 (+)	HPV11−2.5×10^5	4.9×10^5	N.D.	N.D.	N.D.
	HR: 31 (+),	HPV31−4.0×10^4	2.0×10^4	N.D.	N.D.	N.D.
AW 1	**LR: 11 (+++)**, 54 (++), 70 (+), 44 (++)	HPV11−3.3×10^6	6.6×10^6	Yes	N.D.[g]	N.D.
	HR: 59 (+++), 16 (++), 56 (+), 68 (++)	HPV16−2.5×10^5	5.0×10^5	N.D.	N.D.	N.D.
AW 2	**LR:** 61 (+)	HPV16−2.1×10^7	4.2×10^7	No	2.9×10^4	7.2×10^2
	HR: 16 (+++), 35 (++)					
AW 3	**LR:** 54 (++), 6 (+++)	HPV16−2.5×10^5	4.9×10^5	N.D.	N.D.	N.D.
	HR: 16 (++)					
AW 4	**LR:** 54 (++)	HPV16−3.9×10^8	7.9×10^8	Yes	1.4×10^5	2.7×10^3
	HR: 16 (+++), 33 (++), 39 (+++)					
AW 5	**HR: 16 (++), 31 (++)**, 53 (++)	HPV16−5.0×10^5	1.0×10^6	N.D.	N.D.	N.D.
		HPV31−3.3×10^5	6.6×10^5	N.D.	N.D.	N.D.
AW 6	**LR:** 61 (++)	HPV16−3.0×10^6	6.0×10^6	N.D.	N.D.	N.D.
	HR:16 (+++), 35 (++)					
AW 7	**LR:** 61 (++)	HPV16−1.6×10^6	3.3×10^6	N.D.	N.D.	N.D.
	HR: 16 (+++), 35 (+++)					

[a] Recurrent respiratory papilloma (RRP), Anal wart (AW).
[b] Determined by Anyplex II HPV28 detection (Seegene), LR (low risk), HR (high risk).
[c] Based on total recovery volume of 0.5 mL.
[d] Based on RNA-ISH.
[e] Based on total VGE load/focus-forming units (FFU) measured by RNA ISH data (Fig. 4 panels i, j, m, n and data not shown).
[f] Not determined (N.D.).
[g] Stock was depleted with the RT-qPCR assay.

by well-characterised L1 genotype-specific monoclonal antibodies [29]. HPV infections fail to produce cytopathic effects in cultured cells and thus cannot be measured by quantitative viral plaques or quantal assays. HPV infections are measured *via* detection of spliced E1^E4 mRNAs, which are the most abundant HPV transcripts detected post infection (p.i.) [12,31,32]. E1^E4 mRNAs are undetectable in the first hours p.i., their levels rise over 48h p.i. [13,33], and detection is abrogated by antibody-mediated neutralisation of virions [12,34−36]. Therefore, HaCaT cells were inoculated with viral stocks and E1^E4

transcripts were quantified after 5 min (*T* = 0 h), 24 h, or 48 h. In parallel, virus stocks were incubated with genotype-specific neutralising antibodies prior to inoculation and infection for 48 h. Infection for 48 h allows maximal viral transcription without permitting cells to become overly confluent, which we show suppresses early HPV transcription [37]. Each QV stock and the xenograft HPV11 virus exposures were devoid of detectable E1^E4 transcripts at 0 h p.i., and as expected, HPV mRNA levels increased substantially after 48 h p.i. (Fig. 2). Mock infected controls were

Table 2
Characteristics of HPV virus preparations.

Virus Genotype (stock ID)	Source	Final Virion Isolation[a]	Physical titre (VGE/mL)	Antibody neutralisation	Dynamic range of infection	VGE per FFU[h]
HPV11 (10.10)	293T QV	CsCl density gradient	1.2×10^{10}	99%	3-4 $\log_{10}$	3.8×10^4
HPV11	Xenografts[b]	Debris Sedimentation at 11,000 x g[c]	2.9×10^9	98%	3 $\log_{10}$	5.0×10^4
HPV16 (12.35)	293T QV	CsCl density gradient	3.6×10^{11}	99%	4-5 $\log_{10}$	3.8×10^5
HPV16 (12.40)	293T QV	CsCl density gradient	1.5×10^{11}	100%	4-5 $\log_{10}$	N.D.
HPV16	HCK 16-8 Rafts	Crude Virus Preparation (CVP)[d,e]	1.0×10^9	53%	N.D.[g]	no FFU
HPV16 (19.17)	W12-E Rafts	CVP	2.4×10^7	55%	N.D.	no FFU
HPV16 (19.40)	W12-E Rafts	CVP	8.4×10^6	40%	N.D.	no FFU
HPV31 (18.81)	293T QV	CsCl density gradient	3.4×10^{10}	100%	3-4 $\log_{10}$	1.6×10^4
HPV31 (13.02)	CIN-612 9E Rafts	Virion Sedimentation Ultracentrifugation[f]	2.0×10^8	97%	≥5 $\log_{10}$	3.0×10^3

[a] Details of isolation in Methods.
[b] Gift from N. Christensen (PennState College of Medicine).
[c] VirTis homogenisation, supernatant following debris sedimentation at 11,000 x g.
[d] Gift from C. Meyers (PennState College of Medicine).
[e] Dounce homogenisation, low speed debris pelleting per Conway et al. 2009.
[f] BeadBeater homogenisation, high-speed centrifugation, ultracentrifugation at 130,000 x g; based on sedimentation coefficient of 296S−300S for viral DNA-containing particles.
[g] Not determined.
[h] Based on RNA-ISH with FFU related to the total VGE exposed to cells.
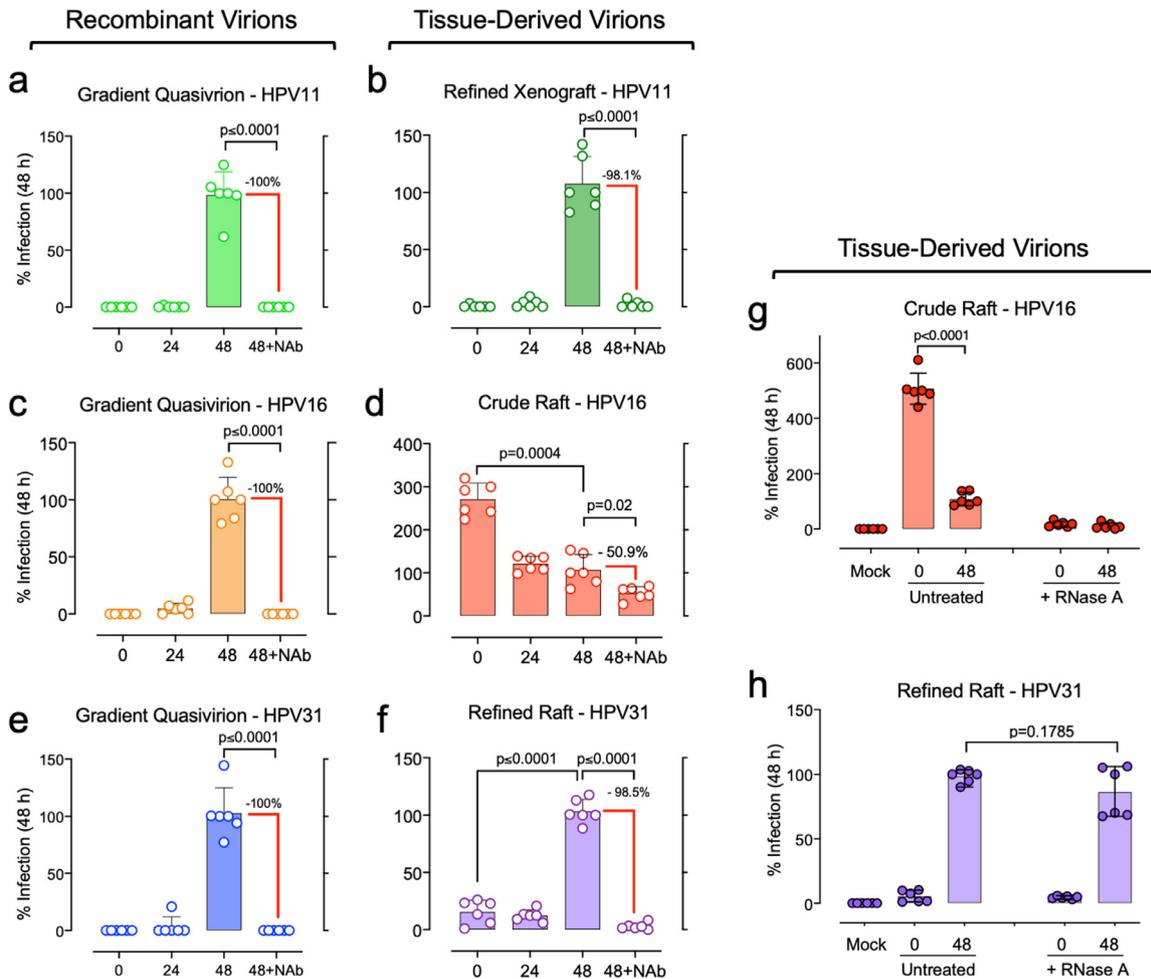
Fig. 2. Infection time course and antibody neutralisation characteristics of HPV virion stocks. Replicate cultures of subconfluent HaCaT cells were incubated with recombinant HPV quasivirions (a, c, e) or tissue-derived HPV preparations (b, d, f). Inocula were exposed to cells for 5 min and were either harvested for total RNA (0h infection), or fresh media were added for infection for 24h or 48 h. A replicate set for each virus stock was incubated with a genotype-specific anti-virus-like particle, monoclonal antibody prior to cell exposure for 48h. The antibodies included: anti-HPV11 clone 11F.G1, anti-HPV16 clone H16.V5, anti-HPV31 clone H31.A6. (g, h) Raft tissue-derived virus stocks were untreated or RNase A treated prior to cell exposure for 0 or 48 h. RNAs were subjected to RT and triplicate qPCR for spliced E1^E4 mRNA quantification (circles); values were normalised to 100% infection at 48 h p.i. Bars represent the mean values of triplicate qPCR reactions from two experimental replicate infections; error bars indicate SD [with paired, two-tailed t-tests performed on indicated values].

consistently negative for E1^E4 targets. The refined, raft-derived HPV31 stock had low levels of E1^E4 mRNAs at 0 h; however, the E1^E4 mRNA levels increased significantly with an average of 10-fold at 48 h p.i., consistent with considerable viral transcription following nuclear viral genome delivery (Fig. 2f). Additionally, each of these virus stocks was neutralised >98% with genotype-specific monoclonal antibodies ($p \leq 0.0001$). Conversely, the HPV16 CVPs yielded anomalous results, inconsistent with all other virion stocks. Not only were relatively high E1^E4 mRNA levels detected immediately after inoculation, but the E1^E4 levels decreased significantly over 24–48 h p.i. ($p = 0.0004$; Fig. 2d). Additionally, the CVP stocks were predominantly resistant to antibody neutralisation (Fig. 2d).

RNase treatment of the crude and refined raft-derived HPV stocks prior to inoculation confirmed that both contained contaminating HPV E1^E4 mRNAs which could be misjudged as infection (Fig. 2g−h). However, RNase treatment of the refined, raft-derived HPV31 stock did not significantly reduce the overall levels of E1^E4 mRNAs detected at 48 h p.i. (Fig. 2h), verifying that E1^E4 detection at 48 h p.i. was a valid measure of infection for this virus isolate. Conversely, RNase treatment of the HPV16 CVP resulted in little to no detection of E1^E4 mRNAs at 48 h p.i. (Fig. 2g), indicating that

contaminating viral RNA in the CVP confounded the infection assay. Taken together, the infection time course studies and the low antibody-mediated neutralisation of HPV infections revealed that the CVPs contained E1^E4 mRNAs that could be inaccurately attributed to infection at 24 h and 48 h p.i. This could explain CVP resistance to the protein crosslinking actions of OPA and glutaraldehyde in the previously reported disinfection studies.

In assessing virus preparation purity by SDS-PAGE, when adequate stocks permitted, virus purity was not an absolute predictor of infectious capacity or susceptibility to neutralising monoclonal antibodies (Suppl. Fig. 1). Infection-validated, xenograft-derived HPV11 was composed of proteins with sizes corresponding to HPV capsid proteins, L1 ($\approx$55kD) and L2 ($\approx$72kD), and cellular histones ($\approx$10−15kD); but numerous other protein species were also present (Suppl. Fig. 1a, lane 6). High-efficiency HPV QV assembly yielded relatively pure preparations with protein sizes consistent with L1, L2 and cellular histones (Suppl. Fig. 1b−d). Limited recovery of HPVs from the raft tissues precluded our ability to both perform infectivity studies and SDS-PAGE characterisation. Yet, we previously reported that neither sedimentation- nor density gradient-ultracentrifugation refinements yielded infection-validated raft-tissue virus preparations with obvious L1 or L2 species amongst the many proteins detected after SDS-PAGE [22].

3.3. Comparison of infectious titres and dose-responsiveness across HPV genotypes and preparations

Studies involving microbial titrations and neutralisation efficacy (e.g., biocides, antibodies) rely on assays that have a broad detection range, ideally over many $\log_{10}$ dilutions [10]. Whereas qPCR approaches are well suited to detect and quantify viral nucleic acid products resulting from infection over a broad range, accurate data interpretation relies on four critical assay performance characteristics: qPCR efficiency, linear dynamic range, limit of detection (LoD) and precision [38]. Studies evaluating HPV disinfection purportedly assayed over a 5 $\log_{10}$-range [7−9]. However, the four essential qPCR characteristics were unreported and there was no evidence that the assays were capable of detecting infection over 5 $\log_{10}$. Therefore, as a prerequisite for systematic evaluation of HPV disinfection, we first determined the qPCR performance characteristics. qPCR calibration curves spanning 10^8−10^0 copies of cloned, E1^E4 cDNA for HPV types 11, 16 and 31 each achieved efficiencies of ≥95%, revealed 7−8 $\log_{10}$ linear dynamic ranges, and LoD were 10^1 or $10^°$ copies per reaction (Fig. 3).

Using internal calibration curves for every assay, we assessed the linear dynamic range of detecting HPV infections over as wide a range of inocula as our virus yields permitted. CVPs were not analysed due to their failure to meet the infection criteria detailed above. Cells were exposed to serial virus dilutions (doses reported in VGE/cell) for 48 h. Between two and six replicate infections were performed for each viral dose, depending upon the amount of virus stock available. Following RT-qPCR, absolute quantities of E1^E4 mRNAs were determined relative to the E1^E4 cDNA qPCR calibration curves (Fig. 3) and revealed a number of important findings. The replicate infections demonstrated consistency (precision) among levels of E1^E4 detected, which decreased as each assay LoD was approached. Whether using tissue-derived virions or QV, simple linear regression analyses indicated a strong association between the virus dose and the quantity of E1^E4 mRNA detected. Parallel dose-response infections with HPV11 xenograft and QV 10.10 stocks yielded R^2 values ≥ 0.97 (Fig. 3a). The highest dynamic ranges achieved for HPV11 were 3−4 $\log_{10}$ due to limited stocks, with similar infectivity when directly comparing xenograft-sourced and QV HPV11 in a single assay (Fig. 3a; Table 2). Dose-response infections with HPV16 and HPV31
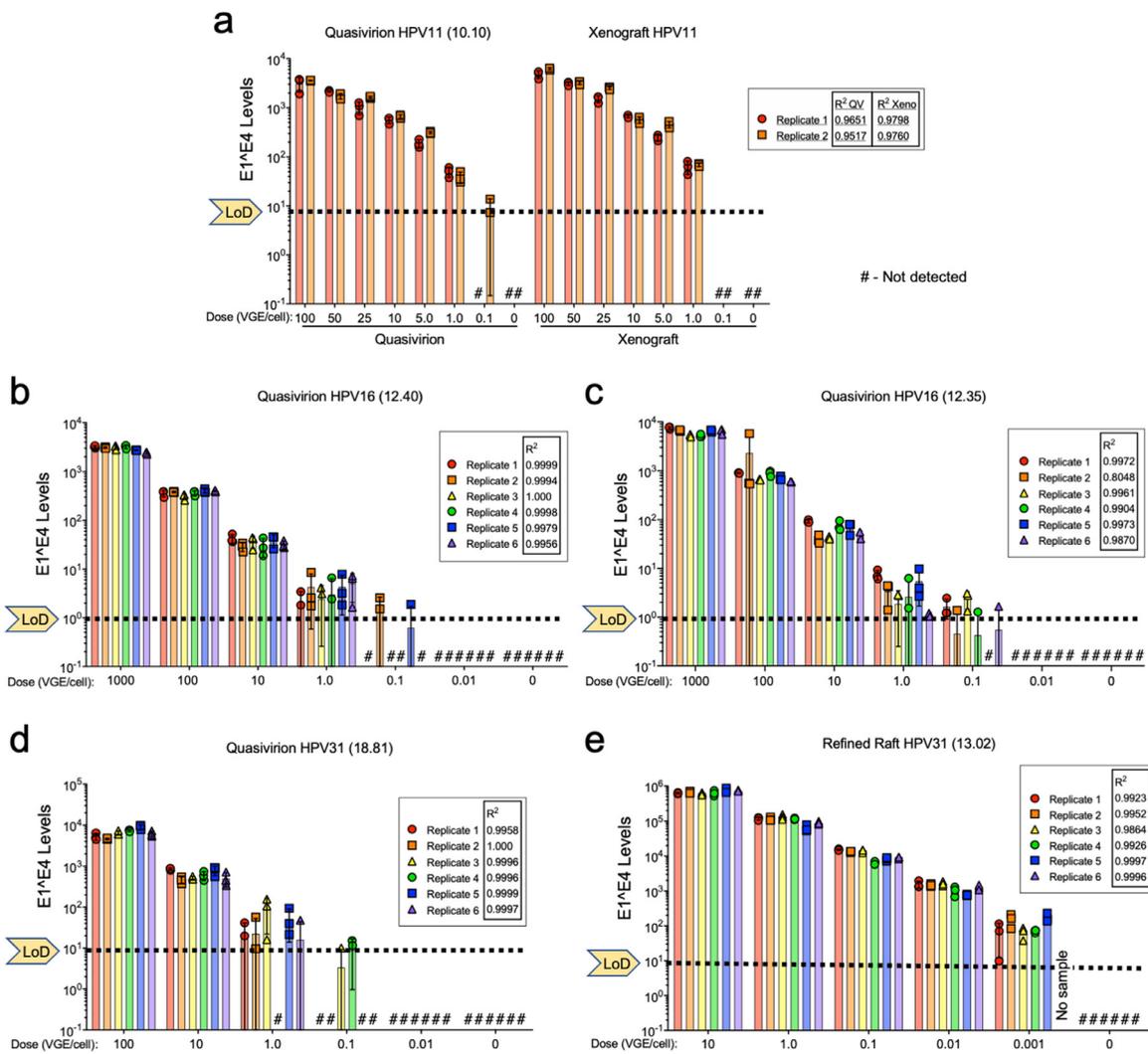
Fig. 3. Linear dose response dynamic range of RT-qPCR quantification and infectivity titration of infectious HPV stocks. HaCaT cells were exposed to serial dilutions of HPV virus stocks that were validated as in Fig. 2. At 48 h p.i., total RNA was extracted and subjected to RT-qPCR with triplicate copy number controls from 10^8 to 10^1 for quantification of HPV E1^E4; ß-actin is a reference target. The y-axis represents the total number of E1^E4 cDNA copies present in triplicate amplifications relative to internal calibration curves attaining ≥95% efficiency. The limit of detection (LoD) for each calibration curve is indicated for each qPCR series. Bars represent the mean of qPCR triplicates (symbols) for each infection replicate; SD error bars are shown. Hashtags (#) specify samples with no detectable E1^E4 targets. Simple linear regression of the mean triplicate values (R^2) is shown for each replicate dilution series.
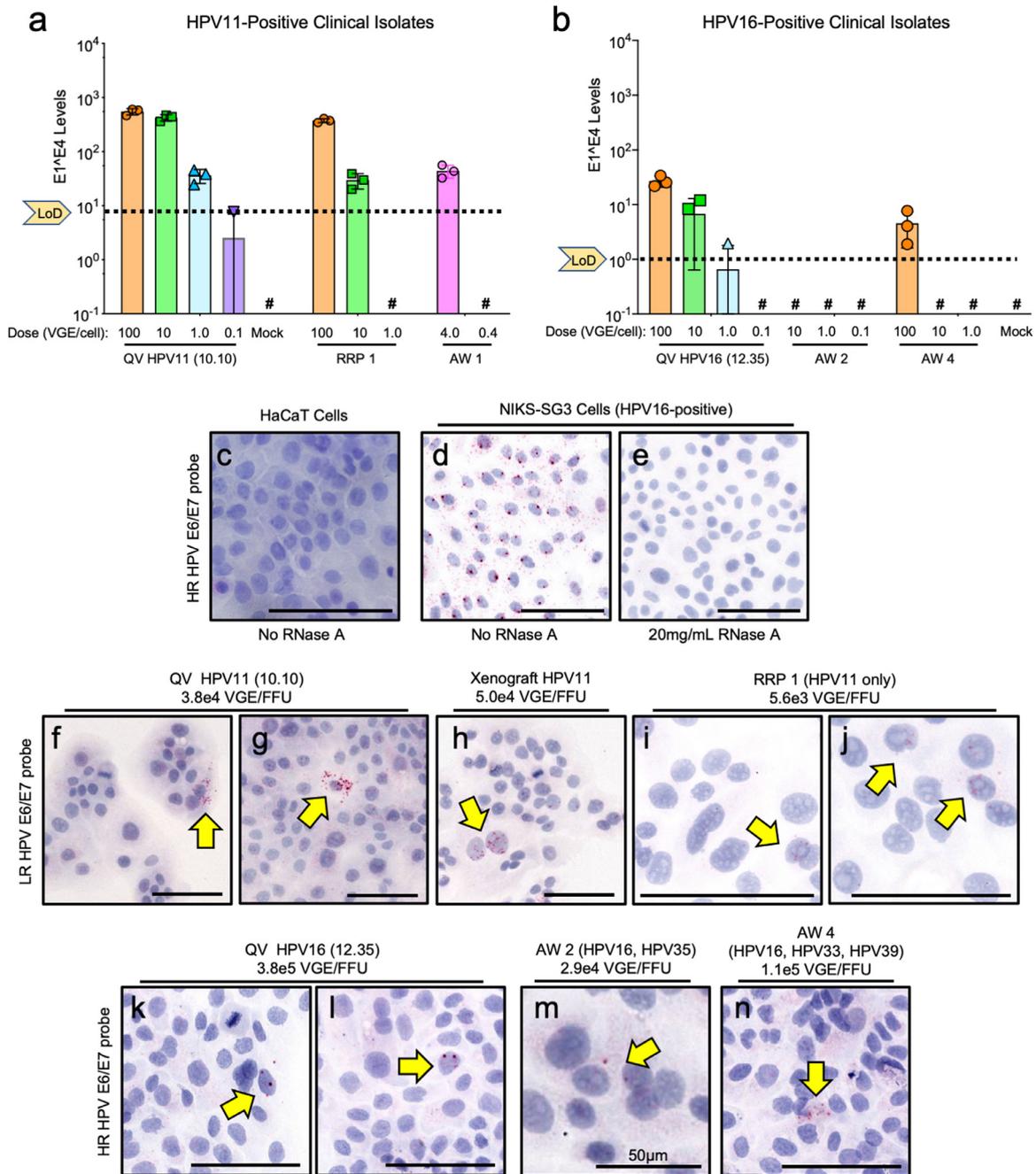
Fig. 4. Infectivity of HPV-positive clinical samples compared with laboratory-sourced HPVs. Genotyped clinical samples from recurrent respiratory papillomas (RRP) and anogenital warts (AW) were freeze-thawed 3x prior to exposure to subconfluent HaCaT cells for 48 h. Xenograft and QV HPV stocks and mock-infected samples were included as positive and negative controls, respectively. (a-b). RT-qPCR with copy number controls from 10^7 to 10^1 for quantification of HPV E1^E4 as in Fig. 2. (c-n) Cells were seeded on chamber slides and exposed to HPV stocks. RNA *in situ* hybridisation (RNA-ISH) was performed for high-risk (HR) and low-risk (LR) HPV genotypes (see Supplemental Information). (c-e) Controls showing specificity of RNA-ISH as labeled. RNA-ISH was performed for LR HPV11 in lab and clinical HPV stocks (f-j) and HR RNA-ISH for HPV16 in lab and clinical HPV stocks (k-n) with focus forming unit (FFU) comparisons to the inocula dose (VGE) for each assay. Arrows point to positive cells containing ≥2 positive puncta indicative of HPV E6/E7 mRNA. Bars = 100 μm unless otherwise noted.

stocks showed strong linearity with R^2 values ≥ 0.98; a linear dynamic infection range of ≥ 4−5 log$_{10}$ was achieved for HPV16 and HPV31 infections (Fig. 3b, c, e; Table 2). The reproducibly low qPCR LoD between 1 and 10 E1^E4 copies per reaction provides a platform to compare HPV virus stocks for relative infectivity based on physical doses of VGE per cell. Most virus stocks yielded reproducible infection at doses ≥1.0 VGE/cell. Refined, raft-derived HPV31 reproducibly demonstrated detectable infection at doses of ≥0.001 VGE/cell (Fig. 3e) for reasons not immediately apparent.

Four clinically-derived HPV-positive samples with the highest physical VGE titres were evaluated for infectivity alongside

characterised QV stocks (Fig. 4). RT-qPCR analyses showed HPV11-positive RRP1 and AW1 were infectious (Fig. 4a). HPV16 infection was detected with AW4, but not the AW2 sample (Fig. 4b). As the clinical isolates were limited and had lower VGE/mL titres than lab-sourced HPV virions (Table 1), we developed a novel, quantitative cell-based infection assay using RNA-ISH that requires one-third fewer cells and lower inoculum volumes. Uninfected HaCaT cells were negative for RNA-ISH signal whereas a persistently HPV16-infected cell line (NIKS-SG3), showed predominantly cytoplasmic detection of HPV mRNAs (Fig. 4c and d). RNase A treatment of NIKS-SG3 cells eliminated the positive ISH signals demonstrating RNA

target specificity (Fig. 4e). Infections were evaluated with doses ranging from 1.0 to 500 VGE/cell revealing isolated cells with positive RNA-ISH signals expressed as an infectious centre, or "focus forming unit" (FFU), relative to the total VGE dose (Fig. 4f−n). Enumeration of ISH-positive cells revealed that HPV11 QV and xenograft stocks had similar RNA-ISH titres at 3.8×10^4 and 5.0×10^4 VGE/FFU, respectively (Fig. 4f−h), consistent with their similar HPV11 RT-qPCR infection profiles (Fig. 3a). HPV11-positive clinical RRP1 was assayed at 5.6×10^3 VGE/FFU (Fig. 4i and j). The AW1 sample was depleted in the RT-qPCR assay, but HPV11-positive RRP5 scored as 7.3×10^3 VGE/FFU (Table 1). Quantification of HPV16 QV infection revealed 3.8×10^5 VGE/FFU (Fig. 4k-l); AW2 and AW4 isolates scored as 2.9×10^4 and 1.6×10^5 VGE/FFU, respectively (Fig. 4m and n). CVP yielded no positive signals in this assay, consistent with their failure in the infection studies noted above. The lower VGE/FFU ratio for clinical isolates compared to HPV16 QV may be attributed to the fact that ISH probes can recognize, in addition to HPV16, other high-risk HPV genotypes that were detected in the clinical samples by DNA typing (Table 1). This novel infectious centre assay revealed that total infectious units isolated from these clinical lesions ranged from 7.1×10^1 to 2.7×10^3 FFU/sample (Table 1). To our knowledge, this is the first assessment of infectious potential of HPVs from clinical lesions.

3.4. Efficacy of disinfectants in vitro across HPV genotypes and isolates

One research group reported aldehyde-based disinfectants to be ineffective in inactivating HPV QV and raft-derived CVP infections in vitro [8,9]. However, as detailed above, we noted several limitations in these studies. Here we provide an independent and critical evaluation using our high-fidelity RT-qPCR assay, which shows that both OPA and hypochlorite effectively disinfect tissue-derived and recombinant HPV preparations. Based on ASTM International guidelines [30], we challenged our infection-validated and well-characterised HPV stocks (Suppl. Fig. 2). Each virus preparation was incubated with either buffer or disinfectants for 12 min at room temperature followed by 10-fold serial dilutions in the appropriate neutraliser for 15 min. Following cell exposure for 48 h, infection levels were quantified (Fig. 5). For each tissue-derived or QV HPV stock, hypochlorite and OPA were each effective disinfectants, consistent with the results of Egawa et al. in a companion manuscript [39]. Hypochlorite completely prevented infection for all HPV stocks tested. Infectivity was undetected after OPA treatment of both xenograft-derived and QV HPV11 virus stocks, although limited virion stock concentrations permitted assessment only over a 2−3 $\log_{10}$ range (Fig. 5a and b). OPA led to a ≥ 4 $\log_{10}$ titer reduction in HPV16 and HPV31 QV infectivity (Fig. 5c and d). Although OPA showed lower effectiveness against refined raft-derived HPV31 infectivity (≥ 3 $\log_{10}$ titer reduction), its efficacy was similar to or slightly better than that of neutralising antibody in direct comparisons (Fig. 5e and f). We cannot rule out the possibility that residual contaminating viral RNA in the raft HPV31 stock is responsible for the OPA- and antibody-resistant E1^E4 signals remaining after treatment. Nevertheless, our data strongly indicate that HPVs are sensitive to disinfection by OPA and hypochlorite.

Control experiments evaluated viral inhibitory effects of neutraliser alone and of neutralised disinfectant compared to untreated virions. OPA inactivated with 1% glycine had a limited inhibitory effect on HPV infection, with the largest inhibition (1.21-1.99 $\log_{10}$) in xenograft and QV HPV11 virus stocks (Fig. 5a and b). Increasing from 1% to 7% glycine appeared to neutralise OPA more effectively (Fig. 5d−f). Inactivated hypochlorite had minimal effects (<0.6 $\log_{10}$) on HPV infectivity, with the exception of HPV16 QV (Fig. 5c). HPV16 QV were similarly inhibited by 2−3 $\log_{10}$ when exposed to hypochlorite neutralised with 2% sodium-thiosulfate (not shown). Simply, this could not be explained by obvious differences in physical characteristics (Suppl. Fig. 1c) or infection phenotypes (Fig. 3b) compared with other virus stocks.

4. Discussion

We determined for the first time to our knowledge the infectious quantities of HPVs from human papillomas and warts that could contaminate medical devices or fomites, and, which lacking adequate decontamination, pose a public health threat for nosocomial transmission. Additionally, using laboratory-produced HPVs at infectious levels that met or exceeded the infectious HPV levels detected from sampling to model clinical exposure, we determined that both OPA and hypochlorite are effective disinfectants in contrast to published reports from another laboratory [7−9].

To quantify the infectious potential of HPV virions shed from clinical lesions, we collected cell exfoliates from the uppermost layers of HPV-induced lesions (Fig. 1a), which are directly relevant to those that may contaminate medical equipment. HPV11 was the predominant genotype in RRPs, whereas HPV16 was the most frequent and high-level genotype in anal warts. Using a novel, cell-based RNA-ISH assay to quantify the infectious potential of clinical HPV isolates, we determined select clinical samples to contain between 75 and 2.7×10^3 infectious units (FFU). HPV genome loads (VGE) outnumbered infectious virions by a factor of 10^3-10^5 (Tables 1 and 2), consistent with reported viral particle-to-infectivity ratios [40]. This emphasises that genome loads should not be assumed to be accurate predictors for infectious virions. Whereas this work was not intended to be a comprehensive evaluation of the viral loads across a population, it does provide an initial assessment of the range of infectious viral loads on the surface of lesions in a small population.

Although aldehyde disinfectants display potent and broad antimicrobial activity and are used in clinical settings worldwide [41], recent reports from a single group indicated that laboratory-produced HPVs are resistant to aldehyde-based disinfection [7−9]. Furthermore, this group found stark differences between raft-derived and recombinant HPVs in their susceptibilities to other disinfectants. Therefore, we evaluated quantitative in vitro HPV infection characteristics, including disinfection susceptibility. Our disinfection experiments employed infectious levels of HPV comparable to those we recovered from clinical lesions. Contrary to previous reports, we show that both tissue-derived and recombinant QV are susceptible to disinfection by both OPA and hypochlorite. These findings are in harmony with the established hierarchy of microbicidal activity [10] based on the non-enveloped nature of HPV virions. Our methodical studies of the infection characteristics of laboratory-produced HPV virions reveal two predominant explanations as to why our OPA disinfection results are in opposition to the previously published reports. First, the prior studies employed crude HPV preparations (CVP) derived from raft tissues [7−9], which we show did not meet common infection criteria of time dependent viral gene expression and strong antibody neutralisation. We demonstrated CVPs were sensitive to RNase treatment, despite being nuclease treated during isolation; this suggests prior studies using CVPs likely measured residual viral RNA contaminating the preparations, rather than authentic infection. Nucleic acids are unlikely to be sensitive to protein crosslinking by aldehydes or antibody-mediated neutralisation. Thus, it is unsurprising that aldehyde-based disinfectants failed to reduce the observed measure of viral mRNAs in aforementioned studies [7−9], or that neutralising antibodies were ineffective on CVPs investigated herein. Conversely, hypochlorite solutions are effective neutralisers of RNA contamination [42] and many viruses [41]. Second, the above-mentioned studies showed no evidence that their RT-qPCR assay had a linear dynamic range capable of detecting infection across the ≥ 4-$\log_{10}$ range they claimed to evaluate. Rather, the data suggested they could only detect infection reduction of ≤ 2-$\log_{10}$ from their untreated control. Lacking essential information, it is
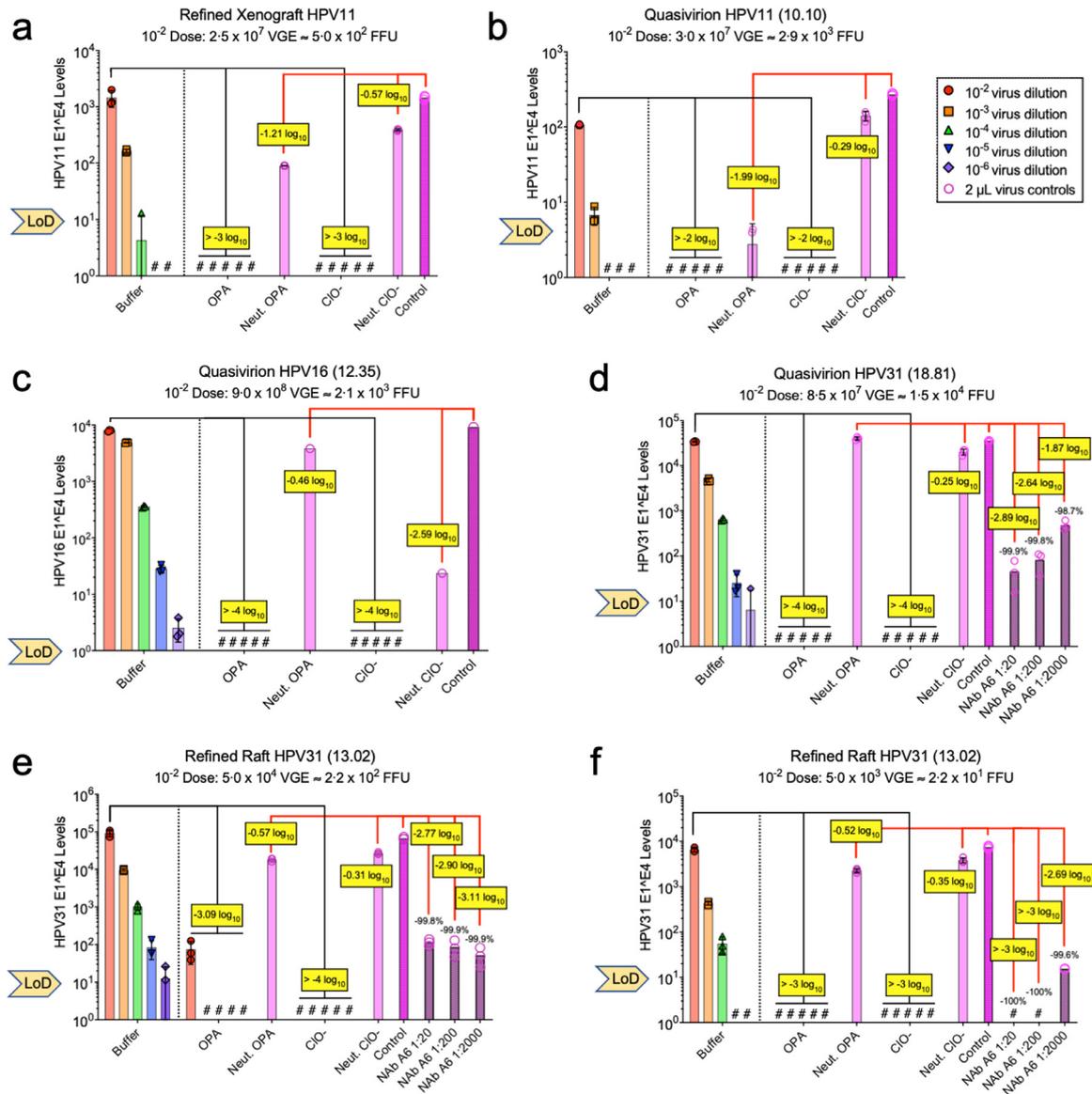
Fig. 5. Disinfection efficiency of OPA and hypochlorite against validated HPV stocks. According to the schematic in Suppl. Fig. 2, 5 μL of virus stocks (infection validated as in Fig. 2) were incubated with buffer as a control or disinfectants (OPA or hypochlorite [ClO-]) for 12min. Virus-disinfectant and virus control (buffer) solutions were neutralised and subject to 10-fold serial dilutions in neutraliser and incubated 15 min (final virus dilutions included 10^{-2}–10^{-6}). Virus was also incubated with neutralised disinfectants (light pink bars) or in cell medium (control; dark pink bars). (a-c) 1% glycine was the OPA neutraliser; (d-f) 7% glycine was the OPA neutraliser. As indicated, virus was incubated with dilutions of a neutralising monoclonal antibody (NAb; striped pink bars). HaCaT cells were exposed to each virus stock for 48 h; the physical load (VGE) and infectious load (FFU) of each virus in the 10^{-2} dilution is indicated. Infectivity levels were determined by RT-qPCR for HPV E1^E4 mRNAs compared to intra-assay, E1^E4 cDNA internal calibration curves; bars represent the mean values of triplicate qPCR reactions. Hashtags (#) specify samples with no detectable E1^E4 targets and the limit of detection (LoD) for each qPCR assays is indicated. Log reduction (yellow boxes) was determined by comparing disinfectant-treated virus to virus treated with buffer (black lines). Neutralised disinfectant and antibody effects on virus infection were compared to untreated control virus (red lines).

impossible to differentiate between their assay LoD and any potential for reduced infectivity. Herein, we employed qPCR calibration curves with a 7-$\log_{10}$ linear dynamic range and a LoD of 10^1 or 10^0 copies per reaction, providing confidence that our qPCR-based infection assay detects infection over the 4- to 5-$\log_{10}$ doses of viral inocula we tested. Since prior reports lacked important controls, any conclusions, particularly those drawn from the testing of disinfectants, should be viewed cautiously.

Our comparison of HPV virus preparations that were isolated by different protocols revealed that purity was not an absolute predictor of infectious potential. Yet, the presence of proteins corresponding in size to L1, L2 and histones correlated with infectivity of xenograft and QV stocks. We have found that QV stocks that lack either L1 or L2 protein species of the appropriate size by SDS-PAGE analyses are infection compromised (data not shown). Although nuclease

treatment of virion stocks can reduce contaminating viral nucleic acids, it could not guarantee removal of HPV mRNAs that might confound infection assays. For future studies of HPV infection and disinfection, we recommend that HPV virion stocks meet bona fide infection criteria, including ≥98% antibody neutralization and time dependent infection detection. Additionally, viral titration assay performance characteristics should be established and reported.

The novel "infectious centre" FFU assay developed herein facilitated titration of clinical virus samples alongside laboratory-derived HPV stocks. While technically more cumbersome than RT-qPCR, advantages of the FFU assay include the need for lower inoculum and the direct enumeration of infected cells. Comparatively, RT-qPCR cannot differentiate between many infected cells expressing low viral RNA levels *versus* few infected cells expressing higher RNA levels. Due to the low virus yields from the clinical samples, we cannot

make definitive conclusions about infectious loads obtained therefrom. However, our physical and infectious virus comparisons strongly suggest that the infectious titres of lab-derived virions used in the disinfection experiments represent similar doses to those from infectious virions that may contact medical devices. It should be emphasized that with any disinfection method, cleaning is of utmost importance; physical removal of microbes and the organic soil that carry them improves the margin of safety contributed by any disinfection method [41]. Therefore, our results, in concert with the companion study by Egawa et al. [39], lead us to conclude that with proper washing of contaminated instruments, OPA and hypochlorite disinfection will minimise potential risk of HPV transmission in associated medical settings.

Contributors

M.A.O., J.Y., M.R., G.E. conceived and designed the study. M.A.O. obtained funding and IRB approvals, and coordinated clinical sampling. V.B., N.A.P., R.T.S. collected the data. M.A.O., V.B., N.A.P., R.T.S. analysed and interpreted the data. M.A.O., V.B., N.A.P. made the figures. A.G.W., E.C.B., R.M. recruited patients, obtained written informed consent, and collected samples. M.A.O., V.B., N.A.P., A.S., J.Y., M.R., G.E. wrote the original manuscript draft. All authors read, revised and approved the manuscript in its final form.

Data sharing statement

The data that support the findings of this study are available from the corresponding author, MAO, upon reasonable request.

Declaration of Competing Interest

M.A.O. reports grants, personal fees, and non-financial support from Advanced Sterilization Products, grants from U.S. National Institutes of Health, during the conduct of the study; A.G.W. reports personal fees and non-financial support from ASCCP, non-financial support from ACOG, non-financial support from Loktal Medical Electronics, personal fees and non-financial support from CSCCP (Chinese Society for colposcopy and cervical pathology, personal fees and non-financial support from Henan People's Regional Hospital, personal fees from Southcentral Foundation (Alaska Native Corp), non-financial support from FECOLSOG (Colombian colposcopy society), non-financial support from COMEGIC (Mexican colposcopy society), non-financial support from Doctors Hospital at Renaissance, non-financial support from MD Anderson, non-financial support from Univ Texas Rio Grande Valley, non-financial support from Global Coalition against Cervical Cancer, non-financial support from AIDS Malignancy Consortium, personal fees and non-financial support from Deaconess Beth Israel Hospital, personal fees and non-financial support from Texas Tech University, personal fees and non-financial support from ABPTGIC (Brazilian colposcopy society), outside the submitted work. V.B., N.A.P, R.T.S., E.C.B. and R.M. have nothing to disclose. A.S., J.Y. and M.R. are employees of Advanced Sterilization Products. G.E. is an employee and shareholder of Johnson & Johnson, which was the parent company for Advanced Sterilization Products and Janssen Pharmaceutica NV at the time the research was conducted.

Acknowledgments

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.ebiom.2020.103165.

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